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STANDARD OPERATING PROCEDURE FOR MUTAGENICITY TESTING USING THE--ETC(U)
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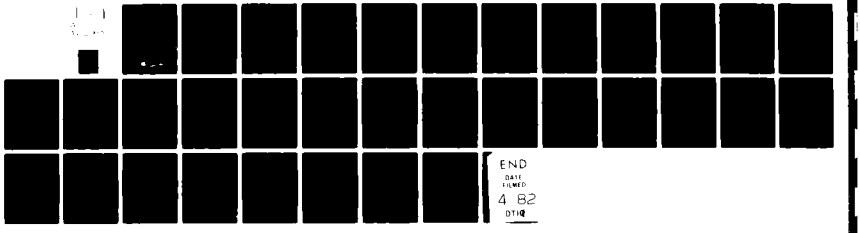

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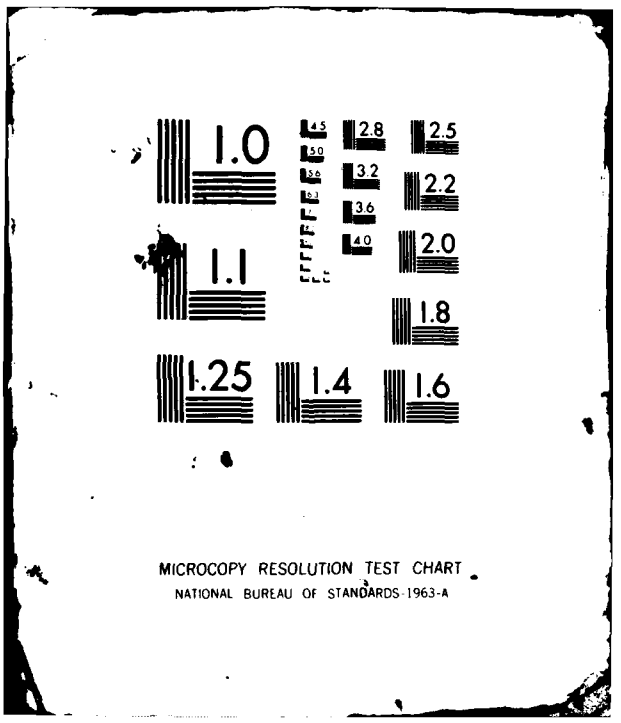
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INSTITUTE REPORT NO. 112

STANDARD OPERATING PROCEDURE for... MUTAGENICITY TESTING USING THE *DROSOPHILA MELANOGASTER* SEX-LINKED RECESSIVE LETHAL ASSAY

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TOXICOLOGY GROUP,
DIVISION OF RESEARCH SUPPORT

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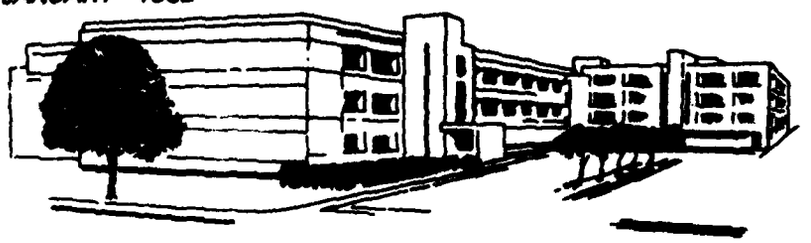
Toxicology Series 29



LETTERMAN ARMY INSTITUTE OF RESEARCH PRESIDIO OF SAN FRANCISCO CALIFORNIA 94129

STANDARD OPERATING PROCEDURE for...
Mutagenicity Testing using the Drosophila melanogaster
Sex-linked Recessive Lethal Assay--Wirtz et al

In conducting the research described in this report, the investigation adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care, Institute of Laboratory Animal Resources, National Research Council.

Joan A Mansfield 13 Jan 1982
(Signature and date)

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The methodology and work schedule followed at Letterman Army Institute of Research for mutagenicity testing of selected materials using the Drosophila melanogaster sex-linked recessive lethal (SLRL) assay are described. Testing procedures were designed to insure compliance with the Food and Drug Administration Good Laboratory Practices regulations. Methodology in the following areas is emphasized: procedures for stock colony maintenance and test insect handling, phenotypic descriptions of Drosophila melanogaster strains used,

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feeding exposure methodology for aqueous and non-water soluble materials, use of positive and negative control compounds, SLRL testing procedures, daily work schedule, data handling and statistical analysis of results.

ABSTRACT

The methodology and work schedule followed at Letterman Army Institute of Research for mutagenicity testing of selected materials using the *Drosophila melanogaster* sex-linked recessive lethal (SLRL) assay are described. Testing procedures were designed to insure compliance with the Food and Drug Administration Good Laboratory Practices regulations. Methodology in the following areas is emphasized: procedures for stock colony maintenance and test insect handling, phenotypic descriptions of *D. melanogaster* strains used, feeding exposure methodology for aqueous and non-water soluble materials, use of positive and negative control compounds, SLRL testing procedures, daily work schedule, data handling and statistical analysis of results.

PREFACE

We would like to express our sincere appreciation to Drs S. Abrahamson and R. Valencia, University of Wisconsin; Dr. G. Schmolesky, Raltech Scientific Services, Inc., Madison, Wisconsin, and Dr. B. Evans and Ms. J. White, SRI-International, Palo Alto, California, for their assistance in establishing our Drosophila Insectary and mutagenicity testing capability.

TABLE OF CONTENTS

Mutagenicity Testing using the Drosophila melanogaster Sex-linked Recessive Lethal Assay

The common fruit fly, Drosophila melanogaster, is an ideal multicellular organism for the rapid toxicologic screening of substances for mutagenicity. Testing procedures using Drosophila have distinct advantages over other systems. The insects have a short generation time, are easily and economically reared in large numbers, can be used to test for inheritable mutations, can detect indirect mutagens without adding activation enzymes, and Drosophila are more complex organisms than the bacteria used in microbial systems (1-7).

An examination of the discriminatory power of different systems using Drosophila reveals that the most sensitive test, which also detects the broadest range of mutagens, is the sex-linked recessive lethal (SLRL) assay (1,2,6,8). This test uses insects of known genotype and detects lethal mutagenic changes in 800-1000 loci on the X-chromosome, representing approximately 20% of the entire genome (1,6). Test results are determined by examining of the second filial (F_2) generation with scoring based on the absence of an expected phenotypic class. To date, most of the research on the mutagenic response of Drosophila to test substances has been conducted using the SLRL test (2,6).

This report describes the specific methodology and work schedule followed in our laboratory for the screening of potential mutagens using the SLRL Assay. Our testing procedures evolved from those currently in use at the laboratories of the Department of Zoology, University of Wisconsin and Raltech Institute, Madison, WI (five on site visits), the Swiss Federal Institute of Technology, Zurich, Switzerland (1), and the University of Leiden, Leiden, Netherlands (1-3).

The methodology varies in different laboratories even though the genetic principles of the test system are identical (1,6). The following procedures were designed to ensure compliance with the Food and Drug Administration Good Laboratory Practices (GLP) regulations (9). This technical report will serve as the primary reference for our future publications in which the SLRL Drosophila melanogaster Test is used.

INSTRUCTIONS

This STANDARD OPERATING PROCEDURE is prepared as a manual/workbook. Use it in the following manner:

- Follow instructions as printed on the left-hand pages for...
  - Stock Colonies and Insect Handling
  - Solubility Methodology
  - Sex-Linked Recessive Lethal Testing
  - Statistical Analysis
- Follow Work Schedule on pages 20 through 25.
- Use blank pages and extra space for notes.

EXPLANATORY INFORMATION relative to the instructions appears on the right-hand pages of each subsection.

* * * * * * * * * * *

PROCEDURE
Materials
Methods
Stock Colonies and Insect Handling

PROCEDURE

Materials

Positive Control Compounds i.e. ethyl methanesulfonate (EMS) (10) (Fisher Scientific, Santa Clara, California) and tris-(2,3-dibromopropyl)-phosphate (TRIS) (11) (INC Pharmaceuticals, Inc., Plainview, NY.)

Drosophila strain, Canton-S (CS), wild type stock, Department of Zoology, University of Wisconsin, Madison, Wisconsin.

Drosophila strain, First Multiple Number Six (FM6), Department of Zoology, University of Wisconsin, Madison, Wisconsin.

LAIR Drosophila Medium, explanation of materials, preparation procedure and their sources are given by Wirtz and Semey (12).

Methods

(SOPs OP-STX-6, OP-STX-8)

Stock Colonies and Insect Handling

- Rear Stock colonies in bottles, 125 ml in volume.
- Anesthetize D. melanogaster with ether designated "Ether for Anesthesia."
- Set up stock colonies on a weekly basis.
- Etherize adult insects for stock colonies and examine for visible mutations, under a dissecting microscope (7 x 30X):
- Destroy all insects if a visible mutant is found in a stock bottle.
- Transfer all insects (30 $\pm$ 10 adults) that appear normal to a bottle containing fresh medium.
- Remove adults 1 to 10 days after setting up the stock bottles.
- Hold colonies as long as needed and autoclave before disposal.

Continued on page 6

EXPLANATORY INFORMATION (Stock Colonies and Insect Handling)

Positive control substances are compounds known to induce lethal mutations in D. melanogaster. Such materials are routinely used for aqueous-based feeding exposure methodology and are normally used at concentrations of 0.1 to 10 mM (13). These concentrations minimize exposure to laboratory personnel and ensure that the SLRL Test System is functioning properly.

All test materials are to be treated as suspected carcinogens as they may have mutagenic or carcinogenic activity, or both. Handling procedures for positive control and test compounds comply with GLP (9) and "The OSHA Cancer Policy" regulations (14).

The strains of D. melanogaster used for these procedures have been used extensively for SLRL testing. The CS strain is a wild-type stock which has been maintained in laboratories for many years. It was chosen for mutagenicity studies because it has shown a relatively low constant spontaneous mutation frequency. The FM6 strain is a laboratory stock containing a homogzygous X-chromosome which carries the phenotypic markers for yellow body (y), bar-shaped eye (B), white eye (w) and several superimposed structural inversions which prevent "crossing over" (exchange of chromosome sections) with homologous non-inverted X-chromosomes.

Collection of CS males by etherization 24 hours after clearing is done. This ensures a fairly uniform age grouping (emergence within the past 4-8 hours). These are the adult males for exposure testing. Collection of FM6 females by etherization 6 hours after clearing is done to ensure virginity.

Media preparation and dispensing procedures for stock colonies and test insects are described by Wirtz and Semey (12).

Continued from page 4. . .

- Clear stock colonies in the morning and collect emerging CS males the following day. Place in a vial (15 adult males/vial maximum) and hold for exposure testing.

- Clear stock colonies in the morning and collect emerging FM6 females approximately 6 hours later.

- Separate virgin FM6 females into groups of 3 and place in vials containing rearing medium (1).

Solubility Methodology

(SOP-OP-STX-7, OP-STX-39)

Solubility Methodology

Determine solubility of materials to be tested by consulting standard references (15,16) and sources of test compounds. If information is not available, conduct laboratory tests to determine solubility.

Note: To determine solubility, attempt to make a 100 mM solution of test material in deionized water. If the compound will not go into solution, decrease the concentration to 10 mM or to 1 mM, if necessary. Depending on the solubility of the solute up to 5% carrier solvent (final concentration) may be used. Mix test chemicals, positive and negative control compounds soluble in water, or the water carrier solvent, with sucrose, glucose, or fructose (1-5%).

- Use water, the ideal test solvent.

- Use dimethyl sulfoxide (DMSO), ethanol, acetic acid or similar agent can be used in low final concentrations (5%) to overcome solubility problems.

- Maintain the pH range so that the flies may feed. Use a buffered solution if necessary.

- Check the pH of this solution with indicating paper. If the pH is above or below the 5 to 9 range, use "Trizma Pre-set pH 7.0 Crystals" (Sigma Chemical Co., St. Louis, Missouri) at the same molar concentration as the test material, to mix up the feeding solution containing 1-5% sucrose, glucose, or fructose.

Note: If the test material is not soluble by the described methodology in the solvents or the exposure methodology is not applicable, eg, gases, consult the principal investigator for alternative methods.

(SOP-OP-STX-7)

Exposure Methodology

- Prepare glass feeding vials (95 x 25mm) by placing one 2.1 cm glass fiber disc (Whatman GF/A) onto the bottom of the empty vial and tamping down a second 2.4 cm disc onto the first.

Continued on page 10

EXPLANATORY INFORMATION (Solubility and Exposure Methodology)

The preferred test concentration for a single dose test is the 72 hour LC_{50} (concentration at which 50% of the flies die after 72 hour exposure). This exposure period of 72 hours was selected since the flies must ingest some of the feeding solution to survive for 72 hours. Feeding concentration and duration of exposure of the test materials are determined by pilot toxicologic and palatalibity studies.

The best route of exposure depends on the biological and chemical properties of the test material. Information about the possible mode of action of a suspected mutagen should be taken into consideration. Also critical to the testing scheme is the stability of the material in the solvent used. The route of exposure may be crucial in the case of indirect mutagens requiring activation by enzyme systems and directly acting chemicals of short half-life. If the above information is unavailable, as is usually the case, experience indicates that it is reasonable to start with testing the possible effects of the compound on spermatogenesis by feeding (1,6,17).

Though the feeding method is preferred, there may be cases in which the use of this method is not possible. Some test materials may be insoluble in any solvent suitable for feeding (although suspensions can be offered, it is doubtful that the flies can ingest any but extremely fine particles). Some test chemicals may also be too toxic for feeding to flies. Since the feeding procedures also subject the flies to contact and possibly inhalation, the toxic effects may be due to the latter exposure rather than ingestion.

For non-water soluble materials, alternative exposure capability using micro-injection, liposome microencapsulation, and lipid oil based solvents are being developed. Other methods available include adult and larval suspension feeding, aerosol treatment ("inhalation"), vaginal douches, and topical application (1,6,17).

DMSO is the most commonly used carrier solvent for mutagenicity testing with Drosophila (6). Recent studies, however, indicate that this solvent can have adverse side-effects, particularly on activation enzyme systems (18). The effects of other carrier solvents on mutagenicity testing have not been examined and should be used only when absolutely necessary. The test concentrations and duration of exposure depends on the solubility, mutagenicity, toxicity, sterility, and palatability of the materials to be examined. The lower limit of feeding concentration is approximately 1 mM. Even potent mutagens can be difficult to detect at lower concentrations since a negative result from a test compound offered at low concentrations may be a false negative. The upper limit of feeding concentration is determined by the solubility of the test material and its toxicity, palatability, and sterility effects.

Continued from page 8. . .

• Apply approximately 0.25 ml of test material to the glass fiber discs with a micropipette. Apply enough material to wet the discs throughly, leaving as little fluid as possible so that the files are not trapped.

• Transfer 24-hour old adult CS males (selected for exposure testing, page 6) to the feeding vial (15 adult males/vial maximum) and plug with a rayon ball.

• Place the feeding vials in a constant temperature incubator (21 $\pm$ 3 C) and every 24 hours transfer the test insects to vials containing fresh material.

• Record the mortality at 24, 48, and 72 hours.

• Increase the concentration of test material, if possible, or lower as necessary to determine the 72-hour LC_{50}.

• Repeat this test as necessary.

(SOP-OP-STX-8)

Sex Linked Recessive Lethal Testing (SLRL)

Preparatory Testing

• Run SLRL test concurrently on an experimental (test) group and positive and negative controls.

• Administer the test (mutagenic activity) compound to the experimental insects (as described on page 8).

• Administer a known mutagen (positive control) at a level which will at least double the background spontaneous mutation rate as observed in the negative control (as described on page 8).

• Administer only the carrier solvent (negative control) (as described on page 8).

• Give each CS male a unique sequential number.

• Allow each numbered insect to mate with 3 virgin FM6 females in a vial containing medium for 3 days (Brood 1). Transfer this male without anesthetization by using a vacuum transfer system (19) to another group of 3 virgin FM6 females, as previously described, for 2 days (Brood 2). Expose this male to a third group of 3 virgin females for 2 days (Brood 3), and then expose the male to a fourth group of 3 virgin females for 3 days (Brood 4).

Lethal Testing

• Allow the daughters of the treated CS males to mate individually to their FM6 male siblings (Table 1).

• Observe the second filial (F_2) flies emerging from the F_1 cross for the presence or absence of normal appearing flies, ie, males that bear the CS X-chromosome with the phenotype of red round eyes.

• Retest suspected lethal mutations from test, from positive control compound, and from negative compound by using an F_3 confirmation cross (Work Schedule, Table 1, page 23). (A lethal mutation in this study is defined as "absence of round red-eyed males"). Do not include mosaic lethals in calculations of mutation frequencies. Enter notation (failure, lethal) on brood card of the male.

EXPLANATORY INFORMATION (SLRL Testing)

The positive control group will assure the investigator that the SLRL test system is functioning properly. The negative control is a measure of the spontaneous mutation frequency.

All progeny of the CS male are assigned the same unique sequential insect number so that progeny of each treated male may be "followed" throughout the 4 broods.

A period of 13 days (3 days exposed to test substance in the vial before ejaculation and 10 days in mating) ensures that the sperm exposed to the test materials are in different stages of development. The males in Brood 1 have mature and nearly mature sperm; Brood 2, primarily spermatids; Brood 3, primarily meiotic-stage sperm; and Brood 4, primarily spermatogonia. The progeny (F_1) from the parental (P_1) can be followed in Table 1, page 23.

The phenotype of males that bear the CS X-chromosome with red, round eyes is of a whole class of flies, one of the four expected classes that is present or absent. The absence of this class in question indicates that mutation to a lethal state has occurred in one or more of the loci on the X-chromosome. The confirmation that lethal mutations have taken place is especially important for weak or supposedly nonmutagenic materials. When only some of the vials of F_3 crosses can be classified as lethal, an F_2 mosaic (or fractional) mutation has occurred (6,17).

A group of chromosomes containing all identical recessive lethals originating from only one mutational event is called a cluster. These may be detected after the inducing either spontaneous lethal or after inducing a lethal by the test material. These are attributed to a mutation in a premeiotic cell which divides one or more times before completing meiosis, and results in more than one mutant chromosome being recovered from the F_2 progeny. The statistical method for detecting and eliminating clusters from raw data is being explored (1,6).

Statistical Analysis

(SOP-OP-STX-10)

Statistical Analysis

➡ Conduct statistical analysis of SLRL mutagenicity data by using Fisher's Exact, Chi-Square, and the Kastenbaum-Bowman Tests (a modification of the Chi-Square Test) (1,6,20,21).

➡ Consult SOP-OP-STX-10 for computerized program of Fisher's Exact Test.

➡ Consult statistical [illegible] for application of Chi-square and Kastenbaum-Bowman [illegible] (20).

EXPLANATORY INFORMATION (Statistical Analysis)

When determining mutagenicity by using the SLRL Test, the size of the groups to be examined depends on several factors, to include the background mutation rate of the stock colonies used, the mutagenic activity of materials tested, the type of statistical test applied, and the level of significance desired.

The historical spontaneous mutation frequency for the CS stock at the University of Wisconsin, Madison laboratory, has been approximately 0.15% and varies from 0.1 to 0.2% (Dr. R. Valencia, personal communication). The background mutation rate in our studies for the same strain has been significantly lower, ie, 0.03% after examining 10,000 X-chromosomes from the negative control insects. In general, lower background mutation frequencies require examination of fewer X-chromosomes for results at the same level of significance.

Test materials with higher mutagenic activity will require exposure and testing of fewer insects than would weaker mutagens. The activity of the test material is usually unknown; however, some materials may be suspect because of results from other mutagenicity tests, chemical structure, or reactivity. Generated data will be examined throughout the testing procedure to see if fewer X-chromosomes can be examined to determine the mutagenic activity of the test material. Doubling of the spontaneous mutation rate, the criterion used by many investigators as an indication that the test material is a weak mutagen, requires the examination of 8000-10,000 X-chromosomes when using stocks with a spontaneous mutation rate of approximately 0.15%. Due to manpower and space limitations we currently divide the testing of each compound into four runs of 2,500 X-chromosomes each. This analysis determines whether or not the mutation frequency in the treated (test) group is significantly larger than that in the appropriate control.

There is some question as to the suitability of the statistical tests currently used for SLRL data analysis. A report addressing this problem, as well as the computer storage and retrival of data, is in preparation (Jederberg, et al, Letterman Army Institute of Research).

Work Schedule

Work Schedule

The schedule for the SLRL Mutagenicity Test is illustrated in Table 2.

Week I

Monday (morning)
- Clear CS and FM6 stock colonies (as described on page 6).
- Collect FM6 females (virgin) (as described on page 6).
- Place three virgin FM6 females in each vial with rearing medium. Repeat this procedure during the week as often as virgin females are needed.

Tuesday (morning)
- Collect CS males (10/vial) from CS stock colonies cleared on Monday.
- Begin adult feeding exposure using the CS males.

Wednesday
Thursday
Friday
- Transfer male insects to vials containing fiber discs saturated with 0.25 ml of solution, when using a water soluble test material. Record mortality counts at 24,48 and 72 hours.

Friday
- Make P_1 matings (Table 1).
- Transfer each treated CS round, red eyed male to a vial containing the three FM6 white bar-eyed females (3-5 days post-pupal eclosion). This is referred to as Brood 1.
- Assign the male a unique sequential number and a computer generated label with the following information attached to each vial: GLP Study Number, when appropriate, run number, unique male number, brood number, starting date, compound code and any applicable notes.

Continued on page 22

EXPLANATORY INFORMATION (Work Schedule)

The work schedule followed in our laboratory for the SLRL testing of materials for which the feeding concentration has been determined is outlined in Table 2.

The matings on Friday of Week I corresponds to Brood 1. On Week II, Monday, Wednesday and Friday matings correspond to Broods 2, 3,and 4 respectively.

The scoring of F_2 and F_3 progeny is accomplished in the following manner; the presence of the CS males indicates a non-lethal (NL) test. If no flies were produced by the mating the test is considered a failure (F). The absence of CS males indicates that a lethal mutation (L) has occurred. The number of NL, F, and L are recorded on the brood card.

Continued from page 20...

Week II	
Monday	• Transfer each treated CS male to a new set of three FM6
Wednesday	virgin females. These matings correspond to broods 2,3
Friday	and 4 respectively. Attach labels to each vial.
Week III	
Monday	• Remove treated CS males from brood 4 after the exposure period on Friday of Week II and dispose of as hazardous waste. (By the latter part of Week III most of the F_1 flies will have emerged).
	• Mate offspring of each of the original P_1 matings, twenty-five daughter (red, bar-eyed); son (white bar-eyed) (Table 1); 1 male x 1 female per vial. Generate a computer brood card (Figure 1).
Week IV	• Continue matings F_1 for broods 2 to 4.
Week V	• Continue F_1 matings, if necessary (late week V the first F_2 flies should be emerging)
	• Begin scoring F_2 flies, 14-21 days after crossing, score each vial for the presence or absence of round red-eyed CS males.
	• Record results on brood cards
	• Do F_3 confirmation cross in the case of apparent lethals (Table 1).
	• Mate three F_3 red bar-eyed females to three FM6 white bar-eyed males in three separate vials for the confirmation cross, 1 male x 1 female per vial.
Week VI	• Continue scoring F_2 progeny, continue F_3 confirmation crosses.
Week VII and VIII	• Score F_3 crosses.

Table 1. Genetic Scheme for Detecting Sex-linked Recessive Lethal Mutation

P_1 mating 1 male 3 females	CS/Y*, treated (red, round-eyed male)		X	FM6/FM6†, virgin (white, bar-eyed female)	
F_1 progeny mating	FM6/CS‡ (red, bar-eyed female)		X	FM6/Y (white, bar-eyed male)	
F_2 progeny	CS/Y" (red, round eyed male)	FM6/CS‡ (red, bar-eyed female)		FM6/Y (white, bar-eye male)	FM6/FM6 (white, bar-eyed female)
Mating of F_2 progeny male female		FM6/CS‡ (red, bar-eyed female)	X	FM6/Y (white, bar-eyed male)	
F_3 progeny	Same as F_2 progeny				

* CS: Canton-S wild-type stock.

† FM6: First Multiple Number 6, and X-chromosomes with a complement of inversions to suppress crossing-over and visible markers (*y B w*)

‡ These females carry the lethal mutation, if it is present.

" Presence of CS males indicates non-lethal; absence indicates a lethal mutation has occurred.

Table 2. Work Schedule for Drosophila melanogaster Sex-linked Recessive Lethal Mutagenicity Test.

WEEK	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
I	1 Clear CS males Collect FM6 virgin females	2 Collect CS males Collect FM6 virgin females CS exposure	3 Renew solutions Collect FM6 virgin females	4 Renew solutions Collect FM6 virgin females	5 Brood 1 matings Collect FM6 virgin females	6	7
II	8 Brood 2 matings Collect FM6 virgin females	9 Collect FM6 virgin females	10 Brood 3 matings Collect FM6 virgin females	11	12 Brood 4 matings Clear Brood 1	13	14
III	15 Clear brood 2	16	17 Clear brood 3	18	19 Clear brood 4 Begin F_1 matings (Br-1)	20	21
IV	22 Begin F_1 matings (Br-2)	23	24 Begin F_1 matings (Br-3)	25	26 Begin F_1 matings (Br-4)	27	28
V	29 F_1 matings continue	30	31	32	33 Begin scoring F_2 (Br-1) F_3 confirmation crosses	34	35
VI	36 Begin scoring F_2 (Br-2) F_3 confirmation crosses	37	38 Begin scoring F_2 (Br-3) F_3 confirmation crosses	39	40 Begin scoring F_2 (Br-4) F_3 confirmation crosses	41	42
VII	43 Continue scoring F_2s all week	44	44	46	47 Begin scoring F_3	48	49
VIII	50 Score F_3s all week	51	52	53	54	55	56

Figure 1, Computer Brood Card

GLP Study No._____

Compound Code

MALE NUMBER BROOD NUMBER STARTING DATE RUN NUMBER

F_2 CROSS MEDIUM BATCH NUMBER _____

DATE:_____ INITIAL:_____

FAILURES:_____ LETHALS:____ NONLETHALS:_____

F_3 CROSS MEDIUM BATCH NUMBER _____

DATE:_____ INITIALS:_____

FAILURES:_____ LETHALS:_____ NONLETHALS:_____

NOTES:_____

_____

CONCLUSION

RECOMMENDATION

REFERENCES

CONCLUSION

The information presented in this manual will serve as a primary reference for future work.

RECOMMENDATION

Refinement of procedures should continue.

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